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Application of zinc formulations of microalgae (Chlorella) in blueberry cultivation

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Abstract

American blueberry is a perennial fruit species, the fruits of which are rich in antioxidants that have a positive effect on human health. During cultivation, it is necessary to pay attention to quality nutrition, protection and agrotechnical measures to achieve the highest quality fruits. This research aims to investigate the effects of foliar application of biopolymer formulations filled with microalgae and zinc sulfate during the cultivation of two blueberry cultivars ('Duke' and 'Aurora') on the quality of the fruit. After the harvesting, in samples of blueberry fruits, some chemical properties were determined (anthocyanins, flavonoids and polyphenols). The results obtained showed that the application of certain bipolymer formulations increased the amount of polyphenols by 17%, flavanoids by 7,2%, and anthocyanins by 26% in the blueberry fruits of selected blubbery cultivars.

Keywords: encapsulation, blueberry, microalgae, microparticles, zinc, foliar

Introduction

Blueberry(*Vaccinium corymbosum* L.) is an increasingly popular food due to its favorable nutritional characteristics. It has numerous positive nutritional and pharmacological properties, such as a high proportion of vitamins, minerals and secondary metabolites; polyphenols and antioxidants. The presence of secondary metabolites in the diet contributes to the normal functioning of the organism, reducing the chance of oxidation processes and the development of numerous diseases. It occurs as a perennial, bushy plant. The main reason for growing this blueberry is its fruit, a berry composed of an exocarp and a mesocarp. It is suitable for consumption in fresh and dried form, freezing and processing (Nikolić et al., 2015). The leading world producers are the USA (294,000 tons), Canada (146,370 tons) and Peru (180,300 tons), while 720 tons of blueberries were grown in Croatia in 2020 (FAOSTAT,2022). The most commonly cultivar are 'Duke', 'Aurora', 'Patriot', 'Bluecrop', 'Nui' and 'Hannah's Choice' (Nikolić et al., 2015). Species of the genus *Vaccinium* began to be collected and experimentally cultivated as early as the beginning of the 18th century (Trehane, 2004), and experienced the greatest commercial success in the period between 1995 and 2005, when the area of total blueberry plantations increased by 90% (Zhao, 2007).

Blueberries require special climatic and environmental conditions that must be ensured for profitable production and the highest quality. Blueberry fruit has a low energy value and is rich in nutritious and bioactive components such as flavonoids, phenolic acids, vitamins and minerals (Nikolić et all, 2015, Nile et all., 2014). It has high antioxidant properties thanks to a large amount of phenolic compounds (Soče, 2019). The antibacterial, antimutagenic, anti-inflammatory and anti-cancer effects of certain active components of blueberry have also been confirmed (Landete, 2012). They have a favorable effect on maintaining the health of the circulatory system and improving vision (Nabavi, 2018). Flavonoids in food are responsible for pigments, aroma, annulation of oxidation and protection against the deactivation of enzymes and vitamins (Yao et al., 2004). The most abundant flavonoids in strawberry fruits are anthocyanins, and it has been proven that blueberry anthocyanins and other polyphenols have beneficial effects on reducing hyperglycemia, body weight and cholesterol accumulation (Roopchand et al., 2013). The nutritional composition of crops, including blueberries, can be improved by appropriate nutrition. One of the ways is the feeding of microalgae, which gives satisfactory results as a supplement in the food industry and agriculture.

According to the systematics of microalgae, they are prokaryotic and eukaryotic photosynthetic microorganisms. Their cells have a simple structure, that ensures rapid and successful growth in unfavorable conditions, which is

why they are found in diverse and extreme ecosystems; deserts, thermal springs and under the ice of Antarctica. (Heimann et all. 2015). Microalgae are attracting attention as a high-potential raw material for biofuel production (Sikirić et al., 2019). They are a potential source of numerous bioactive and energetic substances such as polymers, peptides, fatty acids, carotenoids and sterols. Their photosynthetic activity is high, which is why they perform rapid growth and biomass synthesis (Kim, 2015). They have a lot of potential as sources of carotenoids, vitamins and phenols, and as natural antioxidants, they can cancel lipid peroxidation in food and serve as a natural preservative (Vadlja, 2019). The production of microalgae itself is simple, and high yields are achieved with minimal investment. Microalgae derivatives are completely biodegradable and have no harmful effects on the environment (Čevid, 2016).

Biopolymer formulations of microcapsules have numerous applications; in food, cosmetic and agricultural production. Various bioactive components can be encapsulated and thus ensure their gradual release, and depending on their size, the speed of release can be determined. When it comes to agricultural production, the application of encapsulated bioactive components is a relatively new method of treatment, and their effect is still being tested. Biopolymer formulations of microcapsules can be used as a supplement, which achieves continuous release, i.e. a longer supplement effect (Vinceković et al., 2016 and Vinceković et al., 2017). Polymers for the preparation of biopolymer formulations may include proteins (gelatin, gluten, casein), carbohydrates (starch and its derivatives, gum arabic, xanthan gum, agar, dextran, alginates, cellulose derivatives), waxes and lipids (glycerides of palmitic and stearic fatty acids, acetyl -alcohol, beeswax) and synthetic polymers (polyvinyl alcohol, polyacrylic acid, polystyrene, polyurethanes and polysiloxanes) (Petrović, 2010).

The aim of this study is the folliar application of microcapsules formulations through the process of spraying them over the crown of the shrub so that the plant could absorb nutritiens through the leaves. With foliar application fertilization are 8-20 times greater than nutrient uptake from the soil through the roots, reducing the consumption of the applied agent.

Material and methods

Plant material and experimental design

The experiment was conducted at the blueberry plantation of Fragaria Ltd in Velika Ludina (45°36'10.1"N 16°36'14.8"E) in 2021. The experiment was set up according to the method of randomized block design in four replicates. Each experimental row consisted of 80 blueberry plants (20 in each repetion), and each treatment was applied to 5 plants in each row.

Treatments: The biopolymer formulations filled with microalgae and zinc sulfate were prepared in the laboratory of the Department of Chemistry, Faculty of Agriculture, University of Zagreb. The control treatment was distilled water, the second treatment is a 10% Chlorella algae solution, and the remaining two treatments were two types of microcapsules; sodium alginate microcapsules with zinc sulfate $ZnSO_4 \times 7H_2O$ and sodium alginate microcapsules with both zinc sulfate $ZnSO_4 \times 7H_2O$ and microalgae solution. The microcapsules were prepared using the ionic gelation technique at room temperature by adding a solution of the carrier of the active substance, sodium alginate, with a Büchi-Encapsulator B-390 (BÜCHI Labortechnik AG, Switzerland) into a solution of zinc sulfate heptahydrate (1 mol dm^{-3})(Vinceković et all. 2016).

Each cultivar of blueberries was treated three times (April 16, May 11, and June 1 in 2021). The foliar application was carried out with a manual pressure sprayer (LUX TOOLS Cl), and about 200 mL of the preparation was used per plant. The preparation contained 10 g of microcapsules per plant (200 g per repetition i.e.800 g per treatment. The application solution was prepared by putting 800 g of capsules and 5.2 L of distilled water into the canister so that there would be a total of 6 L of the treatment agent. The doser of the manual pressure sprayer is 3 L, which was enough for 2 rows of application, i.e. repetitions.

Fruit sampling: Ripe fruits were harvested for the 'Duke' on June 21 and 28 and for the 'Aurora' on August 2 and 9, 2021. For each treatment, at each harvest, an average sample of about 500 g was taken. Samples were stored in a refrigerator at the Department of Pomology Faculty of Agriculture until analysis. The dependent variables in each of the analyzes represented the average of the values of 2 measurements (1st harvest and 2nd harvest.).

Analysis of total polyphenols, flavanoids and anthocyanins: Total polyphenols were determined based on the colorimetric reaction of the Folin-Ciocalteu reagent with some reducing reagents (polyphenolic compounds). The absorbance intensity of the resulting blue coloration at 765 nm is determined spectrophotometrically (Ough et all.,

1988), whereby the intensity of the coloration is directly proportional to the proportion of polyphenolic compounds in the tested sample (Singleton et all., 1965). Results are expressed as gallic acid equivalents (GAE) [mg EGAL/100 g FW]. Total flavonoids (Ivanova et al. 2010) are determined spectrophotometrically Results are expressed as quercetin equivalent (QE) [mg QE/100 g FW] at a wavelength of 360 nm. Anthocyanins are determined by the method according to Giusti and Wrolstad (2001). Results are expressed as equivalent of cyanidin-3-glucoside (C3GE) [mg C3GE/100 g FW] at 520 nm and 700 nm. All spectrophotometric measurements were done by Shimadzu UV-1900i spectrophotometer.

Statistical analysis: The data were presented as mean ± standard deviation. Differences between means of examined chemical parameters of blueberry cultivars were compared by the Kolmogorov-Smirnov test and Wilcoxon W test ta at a significance level of 95%, with a risk level of 5%. The data analysis was done in using SPSS Statistics, version 24.0.) were used. Considering the small samples, one of the non-parametric statistical procedures was used to test each hypothesis, although the Kolmogorov-Smirnov test indicates the normality of the distribution of the results in all measurement subjects for the cultivar.

Results and discussion

The results of chemical analyzes of blueberry fruits for phenols can be found in Tables 1 and 2. According to the authors Dongnan et al. (2017) the average amount of polyphenols in the fruits of the 'Duke' is 380 mg/100 g FW. The Mann Whitney U test and the Wilcoxon W test determined a statistically significant difference in the amount of polyphenols in the fruits between the 'Aurora' and 'Duke', and it can be determined with 99% certainty that the fruits of the 'Aurora' have a higher amount of polyphenols compared to the fruits of the 'Duke', with 1% risk (Table 3.).

Table 1. Polyphenols, Flavanoids and Anthocyanins content in blueberries 'Duke'

Treatment	Polyphenols mg EGK/100 g FW		Flavonoids mg QE/100 g FW		Anthocyanins mg C3GE/100 g FW	
	1.harvest	2.harvest	1.harvest	2.harvest	1.harvest	2.harvest
Control	293.82	183.23	205.63	136.61	136.59	69.74
Microalgae solutions	317.59	173.86	217.64	136.64	144.59	71.26
Microcapules zinc	298.98	182.29	221.60	138.44	136.86	72.54
Microcapsules microalgae and zinc	288.01	167.11	209.39	126.83	139.02	65.54

Table 2. Polyphenols, Flavanoids and Anthocyanins content in blueberries 'Aurora'

Treatment	Polyphenols mg EGK/100 g FW		Flavonoids mg QE/100 g FW		Anthocyanins mg C3GE/100 g FW	
	1.harvest	2. harvest	1.harvest	2.harvest	1.harvest	2.harvest
Control	276.52	248.50	184.73	190.75	70.94	66.78
Microalgae solutions	256.19	261.81	197.56	219.64	71.24	73.07
Microcapules zinc	323.79	274.60	218.22	219.28	94.45	81.84
Microcapsules microalgae and zinc	326.30	305.52	229.08	222.41	95.66	91.38

Table 3. Statistical analyzes of polyphenols saturation results concerning cultivar

Cultivar	N	M	C	SD	U	W	p
'Aurora'	16	286.88	286.05	29.15			
'Duke'	16	237.99	240.85	30.56			
Total	32				30.00	166.00	0.00**

Legend: N-sample size; M – arithmetic mean; C- median; SD- standard deviation; U- Mann Whitney U test; W- Wilcoxon W test; p- statistical significance
*Note: ** $p < 0.01$; * $p < 0.05$*

According to the authors Dongnan et al. (2017), the average amount of flavonoids in the fruits of the 'Duke' is 220 mg/100 g^{-} FW. The Mann Whitney U test and the Wilcoxon W test showed a statistically significant difference in the amount of flavonoids in the fruits between the cultivars Aurora' and 'Duke', and it can be determined with 99% certainty that the fruits of the 'Aurora' have a higher amount of flavonoids, compared to the fruit 'Duke', with a 1% risk (Table 4).

Table 4. Statistical analyzes of flavonoid results with regard to cultivar

Cultivar	N	M	C	SD	U	W	p
'Aurora'	16	212.74	206.69	27.31			
'Duke'	16	174.10	174.34	16.64			
Total	32				21.00	157.00	0.00**

Legend: N-sample size; M – arithmetic mean; C- median; SD- standard deviation; U- Mann Whitney U test; W- Wilcoxon W test; p- statistical significance
*Note: ** $p < 0.01$; * $p < 0.05$*

The Mann Whitney U test and the Wilcoxon W test determined a statistically significant difference in the amount of anthocyanins in the fruits between the cultivars 'Aurora' and 'Duke', and it can be determined with 99% certainty that the fruits of the cultivar 'Duke' have a higher amount of anthocyanins compared to the fruits of 'Aurora' varieties, with 1% risk (Table 5).

Table 5. Statistical analyzes of anthocyanin results with regard to cultivarvariety

Cultivar	N	M	C	SD	U	W	p
'Aurora'	16	82.22	77.79	13.26			
'Duke'	16	104.54	105.16	15.69			
Total	32				31,00	167,00	0,00**

Legend: N-sample size; M – arithmetic mean; C- median; SD- standard deviation; U- Mann Whitney U test; W- Wilcoxon W test; p- statistical significance
*Note: ** $p < 0.01$; * $p < 0.05$*

Analysis of variance did not establish a statistically significant difference in the amount of polyphenols in the fruits between treatments, and with 95% confidence, it can be determined that there is no difference in the amount of phenols concerning the different treatments (Table 6). Although the results themselves showed some differences (treatments with capsules with zinc and microalgae in the cultivar 'Aurora'), the sample itself was not large enough to prove significant differences through statistical analysis.

Table 6. Statistical analyzes of polyphenols results concerning the type of treatment.

Treatment	N	M	C	SD	H	p
Control	8	250.27	243.58	30.12		
Microalgae solutions	8	259.84	264.83	34.02		
Microcapsules Zn	8	269.91	273.10	35.73		
Microcapsules Alge/Zn	8	269.72	264.78	53.97		
Total	32				1.55	0.67

Legend: N-sample size; M - arithmetic mean; C - median; SD - standard deviation; H - Kruskal-Wallis test; p-statistical significance.
*Note: ** p < 0.01; * p < 0.05*

The analysis of variance did not establish a statistically significant difference in the amount of flavonoids in the fruits between treatments, and with 95% confidence, it can be determined that there is no significant difference in the amount of flavonoids concerning the different treatments (Table 7).

Table 7. Statistical analyzes of flavanoids results concerning of treatment

Treatment	N	M	C	SD	H	p
Control	8	179.47	176.86	18.19		
Microalgae solutions	8	197.77	195.98	36.50		
Microcapsules Zn	8	199.46	196.57	22.89		
Microcapsules Alge/Zn	8	196.98	191.08	37.58		
Total	32				2,99	0,39

*Legend: N-sample size; M - arithmetic mean; C - median; SD - standard deviation; H - Kruskal-Wallis test; p-statistical significance. Note: ** p < 0.01; * p < 0.05*
*Note: ** p < 0,01; * p < 0,05*

The analysis of variance did not establish a statistically significant difference in the amount of anthocyanins in the fruits between treatments, and with 95% certainty, it can be determined that there is no significant difference in the amount of anthocyanins concerning the different treatments (Table 8).

Table 8. Statistical analyzes of anthocyanin results concerning the type of treatment

Treatment	N	M	C	SD	H	p
Control	8	86.01	80.60	22.75		
Microalgae solutions	8	91.13	81.15	24.51		
Microcapsules Zn	8	96.42	93.84	11.72		
Microcapsules Alge/Zn	8	97.93	97.71	13.02		
Total	32				2,79	0,43

*Legend: N-sample size; M - arithmetic mean; C - median; SD - standard deviation; H - Kruskal-Wallis test; p-statistical significance. Note: ** p < 0.01; * p < 0.05*
*Note: ** p < 0,01; * p < 0,05*

Although the results themselves showed some differences (treatments with capsules with zinc and capsules with zinc and microalgae in the variety 'Aurora'), the sample itself was not large enough to prove significant differences through statistical analysis which could be explained because of the weather extremes through the all 2021.

The conducted research opens up the potential for additional investigations of biopolymer formulations of microalgae because they are understudied and have great potential for application in sustainable fruit productionv

Conclusions

The results of this research showed an increased proportion of total polyphenols, flavanoids and an increased proportion of anthocyanins in blueberry fruits of the 'Aurora' cultivar foliar treated with biopolymer formulations with a solution of zinc sulfate $ZnSO_4 \times 7H_2O$ and biopolymer formulations with a solution of *Chlorella microalgae*.

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